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(54) Title: IMMUNOASSAY FOR DETECTING HCV IgM ANTIBODY

(57) Abstract

An assay for determining the presence of HCV IgM in a test sample. A test sample is contacted with at least one HCV antigen, incubated to form antigen/antibody complexes, and then contacted with an indicator reagent in order to produce a detectable signal. The amount of HCV IgM present in the test sample is proportional to the signal generated. The assay also can include the step of contacting the complexes with an enhancer compound prior to the use of an indicator reagent. Also provided is a test kit useful for performing the assay of the invention.

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IMMUNOASSAY FOR DETECTING HCV IgM ANTIBODY

Background of the Invention

This invention relates generally to Hepatitis C Virus (HCV), and more particularly, relates to the detection of HCV IgM in test samples.

Greater than 90% of cases of transfusion hepatitis worldwide are attributed to non-A, non-B hepatitis (NANBH). The predominant etiological agent of NANBH, termed Hepatitis C virus (HCV), has been cloned. An immunodominant region designated as c-100, encoded by the putative nonstructural (NS)-4 genomic region, has been expressed, purified, and incorporated into immunoassays which are useful in the detection of antibody to HCV in infected test samples. See, for example, Q.-L. Choo et al., Science 244:359-362 (1989); H. J. Alter et al., N. Engl. J. Med. 321:1494-1500 (1989); J. I. Esteban et al., Lancet ii:294-297 (1989); G. Huo et al., Science 244:362-364 (1989); T. Miyamura et al., Proc. Natl. Acad. Sci. USA 87:983-987 (1990); and C. L. Van der Poel et al., Lancet ii:297-298 (1989).

Recently, a study of 20 well-documented cases of post-transfusion NANBH reported that the mean delay to the development of anti-HCV was 21.9 weeks after transfusion and 15 weeks after the onset of hepatitis. Alter et al., supra. Seroconversion occurred after six months in 40% of these cases and at approximately one year in one patient. Similar results have been reported for post-transfusion NANBH by J. I. Esteban et al., supra, and for chimpanzees that were experimentally infected with the Hepatitis C Virus by Y. K. Shimizu et al., Proc. Natl. Acad. Sci. USA 87:6441-6444 (1990). The delay in seroconversion to anti-HCV detected by recombinant c100 antigen may account for its relatively low prevalence (15-30%) in individuals diagnosed with acute NANBH. Thus, anti-HCV-negative cases may actually be caused by HCV infection that does not elicit an immune response detectable by current anti-HCV c-100 assays (the so-called first generation assays).

Whereas previous studies have established HCV as the primary cause of transfusion-associated hepatitis, immunoglobulin (Ig) G antibodies to HCV detected by the HCV c-100 antigen are likely to be

absent during acute infection. Vallari et al. recently have discovered that the addition of recombinant HCV CORE and HCV NS3 (33c) polypeptides to IgG anti-HCV assays significantly reduces the post-transfusion period when anti-HCV is undetected. D. Vallari et al., Proc. Natl. Acad. Sci. USA (1991, submitted for publication). In this study, the earliest IgG response to HCV was detected more frequently against HCV CORE antigen (earliest or coincident in 13 patients) than against either HCV 33c (eight patients) or HCV c-100 (six patients). In addition, HCV IgG antibodies were detected more frequently against either HCV CORE (90.8%) or HCV 33c (80.1%) compared to HCV c-100 (59.7%). A significant correlation was evident between the IgG antibody responses detected by HCV 33c and HCV c-100 antigens, as opposed to the responses observed when either of these putative HCV epitopes was compared to HCV CORE. However, neither HCV 33c, HCV CORE, nor a combination of both, obviate the need for HCV c-100 antigen in anti-HCV IgG assays.

The finding of passive transfer of HCV IgG antibodies directed to several putative HCV epitopes indicates that the presence of these antibodies does not neutralize the virus. Furthermore, it has been shown that patients actively producing high levels of antibodies to HCV CORE transmitted hepatitis to chimpanzees. Alter et al., supra. It is not known at this time whether the corresponding viral antigens are present as an immune complex or sequestered in viral particles. It is known that Hepatitis B virus (HBV) CORE antigen contained within Dane particles and high serum titers of anti-HBV CORE occur in HBV carriers. Additional studies are still required to determine whether antibodies directed to other HCV structural components, such as HCV putative envelope protein(s), confer protection against the HCV virus. However, our present understanding of HCV serology points out that individuals who possess antibodies to HCV are likely to be infectious. This notion also is supported by reports that > 40% of anti-HCV c100-positive sera have detectable HCV RNA. A. J. Weiner et al., Lancet 336:695 (1990).

A recent report indicates that institution of blood screening for anti-HCV (c-100) would prevent approximately half of the cases of transfusion-associated hepatitis. J. I. Esteban et al., N. Eng. J. Med. 323:1107-1112 (1990). The "second generation" of anti-HCV screening assays under

development, which employ HCV CORE, HCV 33c and HCV c-100 antigens, should substantially increase the sensitivity for detection of HCV exposure. This increased sensitivity should not only further reduce the risk of transfusion-associated hepatitis, but potentially provide a clearer understanding of the epidemiology of community-acquired NANBH.

Although it is recognized that markers for HCV IgM should exist, the identification of these markers and their usefulness in diagnosis of HCV infections has been unclear. Theoretically, it has been postulated that the ability to detect HCV IgM in test samples could provide an earlier diagnosis than the detection of HCV IgG in test samples, since it is believed that IgM appears first, before IgG, in an individual's response against infection. We have discovered that numerous false reactive results may occur when using assays designed to detect the presence of IgM. We recently determined that a majority of these false reactions occurred due to the presence of Rheumatoid-like factors along with HCV IgG, and not to the presence of IgM, in the test sample. Thus, although the need exists for an assay to determine the presence of IgM, currently available assays do not appear to offer reliable, specific results. It therefore would be advantageous to provide an immunoassay to sensitively and specifically detect the presence of HCV IgM in test samples, and thus to provide a means for determining acute infection due to NANBH. The detection of IgM could also be useful to measure response to therapy, to monitor the response to vaccines, or to assess the reactivation of viral replication, reinfection, or exacerbation of HCV induced liver disease. We have discovered that the detection of HCV IgM antibody against the HCV putative capsid (CORE) protein provides such a marker of post-transfusion HCV infection. We also have discovered a novel immunoassay which sensitively and specifically can detect the presence of anti-HCV IgM antibody in a test sample, and thus provides a useful acute marker of HCV infection.

Summary of the Invention

The present invention provides an assay for determining the presence and/or amount of IgM to Hepatitis C Virus (HCV) which may be present in a test sample. Anti-HCV CORE IgM antibody is shown to be

useful as an acute marker of infection. The assay comprises subjecting the test sample to conditions sufficient to block the effect, if any, of rheumatoid factor-like substances which may be present in the test sample and contacting the test sample with at least one HCV antigen selected from the group consisting of HCV CORE, HCV 33c and HCV c-100 and incubating the resultant mixture for a time and under conditions sufficient to form antigen/antibody complexes. These complexes then are contacted with an indicator reagent comprising a signal generating compound conjugated to an HCV specific binding member and the resultant mixture is incubated for a time and under conditions sufficient for a reaction to occur. The signal generated is an indication of the presence and/or amount of HCV IgM present in the test sample. The amount of HCV IgM present in the test sample is proportional to the generated signal.

The invention further provides an assay for determining the presence and/or amount of IgM to Hepatitis C Virus (HCV) which may be present in a test sample which comprises subjecting the test sample to conditions sufficient to block the effect, if any, of rheumatoid factor-like substances which may be present in the test sample and contacting the test sample with at least one HCV antigen selected from the group consisting of HCV CORE, HCV 33c and HCV c-100 and incubating the resultant mixture for a time and under conditions sufficient to form antigen/antibody complexes. These complexes then are contacted with a probe comprising an enhancer compound conjugated to mammalian anti-human IgM to form a second mixture reaction product. The second mixture reaction product is contacted with an indicator reagent comprising a signal generating compound conjugated to an enhancer compound binding member and the resultant mixture is incubated for a time and under conditions sufficient to form an indicator reagent reaction product. The presence and amount of HCV IgM is determined by detecting the signal generated as an indication of the presence and/or amount of HCV IgM present in the test sample. The amount of HCV IgM present in the test sample is proportional to the signal generated.

Other assay configurations to determine the presence and/or amount of HCV IgM in a test sample also are provided. Thus, another

assay comprises subjecting the test sample to conditions sufficient to block the effect, if any, of rheumatoid factor-like substances which may be present in the test sample, contacting the test sample with anti-human IgM, and incubating the resultant mixture for a time and under conditions sufficient to form resultant complexes. These complexes then are contacted with an indicator reagent comprising a signal generating compound conjugated to HCV antigen comprising at least HCV CORE antigen and the resultant mixture is incubated for a time and under conditions sufficient for a reaction to occur. The presence and amount of HCV IgM is determined by detecting the signal generated as an indication of the presence and/or amount of HCV IgM present in the test sample. The amount of HCV IgM present in the test sample is proportional to the signal generated.

Yet another assay configuration comprises subjecting the test sample to conditions sufficient to block the effect, if any, of rheumatoid factor-like substances which may be present in the test sample, contacting the test sample with mammalian anti-human IgM and incubating the resultant mixture for a time and under conditions sufficient to form antigen/antibody complexes. These complexes then are contacted with a probe comprising an enhancer compound conjugated to HCV antigen comprising at least HCV CORE antigen to form a second mixture reaction product. Then, the second mixture reaction product is contacted with an indicator reagent comprising a signal generating compound conjugated to an enhancer compound binding member and the resultant mixture is incubated for a time and under conditions sufficient for an indicator reagent reaction product. The presence and amount of HCV IgM is determined by detecting the signal generated as an indication of the presence and/or amount of HCV IgM present in the test sample. The amount of HCV IgM present in the test sample is proportional to the signal generated.

Still another assay format comprises subjecting the test sample to conditions sufficient to block the effect, if any, of rheumatoid factor-like substances which may be present in the test sample and contacting the test sample with anti-human IgM, and incubating the resultant mixture for a time and under conditions sufficient to form resultant complexes.

The complexes then are contacted with HCV antigen comprising at least HCV CORE antigen and the resultant mixture is incubated for a time and under conditions sufficient for a reaction product to form. The reaction product is contacted with an indictor reagent comprising anti-HCV CORE antigen conjugated to a signal generating compound, and incubated for a time and under conditions for a reaction to occur. The presence and amount of HCV IgM is determined by detecting the signal generated as an indication of the presence and/or amount of HCV IgM present in the test sample. The amount of HCV IgM present in the test sample is proportional to the signal generated.

The assays described herein can include a solid phase to which HCV antigen is attached. The solid phase selected can include polymeric or glass beads, nitrocellulose, microparticles, wells of a reaction tray, test tubes and magnetic beads. The signal generating compound can include an enzyme, a luminescent compound, a chromogen, a radioactive element and a chemiluminescent compound. Examples of enzymes include alkaline phosphatase, horseradish peroxidase and beta-galactosidase. Examples of enhancer compounds include biotin, anti-biotin and avidin. Examples of enhancer compound binding members include biotin, anti-biotin and avidin. In order to block the effects of rheumatoid factor-like substances, the test sample is subjected to conditions sufficient to block the effect of rheumatoid factor-like substances. These conditions comprise contacting the test sample with a quantity of anti-human IgG to form a mixture, and incubating the mixture for a time and under conditions sufficient to form a reaction mixture product substantially free of rheumatoid factor-like substance.

The present invention also includes a test kit useful for determining the presence and/or amount of HCV IgM in a test sample. Such a test kit comprises a container containing at least one HCV antigen selected from the group consisting of HCV CORE, HCV 33c and HCV c-100, a container containing an amount of anti-human IgG, and a container containing an amount of mammalian anti-human IgM. The kit also can include HCV antigen attached to a solid phase such as polymeric or glass beads, nitrocellulose, microparticles, wells of a reaction tray, test tubes and magnetic beads. The mammalian anti-

human IgM can be attached to a signal generating compound. The signal generating compound can be an enzyme, a luminescent compound, a chromogen, a radioactive element and a chemiluminescent compound. Alternatively, the mammalian anti-human IgM can be attached to an enhancer compound. Examples of enhancer compounds include biotin, anti-biotin and avidin. Further, a signal generating compound can be attached to an enhancer compound binding member. Examples of enhancer compound binding members include biotin, anti-biotin and avidin.

Brief Description of the Drawings

FIG. 1 is a map of the HCV GENOME representing the non-structural (NS) genes and the structural genes, CORE (C) and envelope (E).

FIG. 2 is a graph showing the effect of Rheumatoid Factor on an HCV IgG reactive sample (No. 170) in the HCV IgM assay test, in which ΔDR is plotted against IU/ml of RF. A solid line between closed triangles is the line of anti-HCV 33c, while a solid line between open circles is the line of anti-HCV CORE.

FIG 3 is a graph of the IgM activity of sample No. 1010, wherein DR readings are plotted against the fraction number. A solid line between open squares indicates anti-CKS-CORE, a solid line between "+" indicates anti-33c, and a solid line between open diamonds indicates anti-c-100.

FIG. 4 is a graph of the IgG activity of sample No. 1010 wherein DR readings are plotted against the fraction number. A solid line between open squares indicates anti-CKS-CORE, a solid line between "+" indicates anti-33c, and a solid line between open diamonds indicates anti-c-100.

Detailed Description of the Invention

The present invention provides an immunoassay which utilizes specific binding members. A "specific binding member," as used herein, is a member of a specific binding pair. That is, two different molecules where one of the molecules through chemical or physical means specifically binds to the second molecule. Therefore, in addition to

antigen and antibody specific binding pairs of common immunoassays, other specific binding pairs can include biotin and avidin, carbohydrates and lectins, complementary nucleotide sequences, effector and receptor molecules, cofactors and enzymes, enzyme inhibitors and enzymes, and the like. Furthermore, specific binding pairs can include members that are analogs of the original specific binding members, for example, an analyte-analog. Immunoreactive specific binding members include antigens, antigen fragments, antibodies and antibody fragments, both monoclonal and polyclonal, and complexes thereof, including those formed by recombinant DNA molecules. The term "hapten", as used herein, refers to a partial antigen or non-protein binding member which is capable of binding to an antibody, but which is not capable of eliciting antibody formation unless coupled to a carrier protein.

A "capture reagent", as used herein, refers to an unlabeled specific binding member which is specific either for the analyte as in a sandwich assay, for the indicator reagent or analyte as in a competitive assay, or for an ancillary specific binding member, which itself is specific for the analyte, as in an indirect assay. The capture reagent can be directly or indirectly bound to a solid phase material before the performance of the assay or during the performance of the assay, thereby enabling the separation of immobilized complexes from the test sample.

Test samples which can be tested by the methods of the present invention described herein include human and animal body fluids such as whole blood, serum, plasma, cerebrospinal fluid, urine, biological fluids such as cell culture supernatants, tissue specimens and cell specimens.

An enhancer can be used to detect the generated signal in the assay. By "enhancer" is meant a moiety which can bolster a signal generated in an immunoassay, thereby amplifying the generated signal. Several methods of enhancing and amplifying a signal generated in an immunoassay are known in the art. Also, the use of a signal enhancer such as the use of avidin-biotin also is known. For example, U. S. Patent No. 4,228,237 to Hevey et al. describes the use of a biotin labelled specific binding substance for a ligand used in a method which also employs an

enzyme labelled with avidin. The use of a biotin-anti-biotin system is described in U. S. Patent Application Serial No. 608,849 filed May 10, 1984, which enjoys common ownership and is incorporated herein by reference (published on November 13, 1985 as European Patent Application No. 160,900).

The term "probe," as used herein, means a member of the specific binding pair attached to an "enhancer" compound. An "enhancer" compound can be any compound used in the assay which can enhance the signal generated by the signal generating compound. Thus, enhancer compounds include haptens such as biotin, and also include fluorescein, di-nitrophenol, and the like.

The indicator reagent comprises a signal generating compound (label) which is capable of generating a measurable signal detectable by external means conjugated (attached) to a specific binding member. "Specific binding member," as used herein, means a member of a specific binding pair. That is, two different molecules where one of the molecules through chemical or physical means specifically binds to the second molecule. In addition to being an antibody member of a specific binding pair for HCV, the indicator reagent also can be a member of any specific binding pair, including either hapten-anti-hapten systems such as biotin or anti-biotin, avidin or biotin, a carbohydrate or a lectin, a complementary nucleotide sequence, an effector or a receptor molecule, an enzyme cofactor and an enzyme, an enzyme inhibitor or an enzyme, and the like. An immunoreactive specific binding member can be an antibody, an antigen, or an antibody/antigen complex that is capable of binding either to HCV as in a sandwich assay, to the capture reagent as in a competitive assay, or to the ancillary specific binding member as in an indirect assay. Thus, if an enhancer is utilized in the assay, the indicator reagent comprises a signal generating compound conjugated to an enhancer-specific compound (enhancer compound binding member), such as biotin or anti-biotin, avidin or biotin, and others known to those skilled in the art. For example, if the enhancer compound utilized is biotin, then anti-biotin, or avidin, can be used as the enhancer-specific compound.

The various signal generating compounds (labels) contemplated include a chromogen such as bromo-chloro-indole-phosphate (BCIP), catalysts such as enzymes, luminescent compounds such as fluorescein and rhodamine, chemiluminescent compounds such as acridinium, phenanthridinium or 1,2-dioxetane compounds, radioactive elements, and direct visual labels. Examples of enzymes include alkaline phosphatase, horseradish peroxidase, beta-galactosidase, and the like. The selection of a particular label is not critical, but it will be capable of producing a signal either by itself or in conjunction with one or more additional substances, such as the use of enzyme substrates when enzymes are employed as the signal generating compound.

It is contemplated that the reagent employed for the assay can be provided in the form of a kit with one or more containers such as vials or bottles, with each container containing a separate reagent such as a monoclonal antibody, or a cocktail of monoclonal antibodies, employed in the assay.

The assay configuration may involve the use of a solid phase in performance of the present invention. A "solid phase", as used herein, refers to any material which is insoluble, or can be made insoluble by a subsequent reaction. The solid phase can be chosen for its intrinsic ability to attract and immobilize the capture reagent. Alternatively, the solid phase can retain an additional receptor which has the ability to attract and immobilize the capture reagent. The additional receptor can include a charged substance that is oppositely charged with respect to the capture reagent itself or to a charged substance conjugated to the capture reagent. As yet another alternative, the receptor molecule can be any specific binding member which is immobilized upon the solid phase and which has the ability to immobilize the capture reagent through a specific binding reaction. The receptor molecule enables the indirect binding of the capture reagent to a solid phase material before the performance of the assay or during the performance of the assay. If an assay device is utilized to perform the assays of the present invention, it can have many configurations, several of which are dependent upon the material chosen as the solid phase. For example, the solid phase can include any suitable porous material. By "porous" is meant that the material is one through

which the test sample can easily pass and includes both bibulous and non-bibulous solid phase materials. In the present invention, the solid phase can include a fiberglass, cellulose, or nylon pad for use in a pour and flow-through assay device having one or more layers containing one or more of the assay reagents; a dipstick for a dip and read assay; a test strip for wicking (e.g., paper) or thin layer chromatographic or capillary action (e.g., nitrocellulose) techniques; or other porous or open pore materials well known to those skilled in the art (e.g., polyethylene sheet material). The solid phase, however, is not limited to porous materials. The solid phase can also comprise polymeric or glass beads, microparticles, tubes, sheets, plates, slides, wells, tapes, test tubes, or the like, or any other material which has an intrinsic charge or which can retain a charged substance.

Natural, synthetic, or naturally occurring materials that are synthetically modified, can be used as a solid phase including polysaccharides, e.g., cellulose materials such as paper and cellulose derivatives such as cellulose acetate and nitrocellulose; silica; inorganic materials such as deactivated alumina, diatomaceous earth, $MgSO_4$, or other inorganic finely divided material uniformly dispersed in a porous polymer matrix, with polymers such as vinyl chloride, vinyl chloride-propylene copolymer, and vinyl chloride-vinyl acetate copolymer; cloth, both naturally occurring (e.g., cotton) and synthetic (e.g., nylon); porous gels such as silica gel, agarose, dextran, and gelatin; polymeric films such as polyacrilamide; and the like. The solid phase should have reasonable strength or strength can be provided by means of a support, and it should not interfere with the production of a detectable signal.

Preferred solid phase materials for flow-through assay devices include filter paper such as a porous fiberglass material or other fiber matrix materials. The thickness of such material is not critical, and will be a matter of choice, largely based upon the properties of the sample or analyte being assayed, such as the fluidity of the test sample.

To change or enhance the intrinsic charge of the solid phase, a charged substance can be coated directly to the material or onto microparticles which are then retained by a solid phase support material.

Alternatively, microparticles can serve as the solid phase, by being retained in a column or being suspended in the mixture of soluble reagents and test sample, or the particles themselves can be retained and immobilized by a solid phase support material. By "retained and immobilized" is meant that the particles on or in the support material are not capable of substantial movement to positions elsewhere within the support material. The particles can be selected by one skilled in the art from any suitable type of particulate material and include those composed of polystyrene, polymethylacrylate, polypropylene, latex, polytetrafluoroethylene, polyacrylonitrile, polycarbonate, or similar materials. The size of the particles is not critical, although it is preferred that the average diameter of the particles be smaller than the average pore size of the support material being used.

Solid supports are known to those in the art and include the walls of wells of a reaction tray, test tubes, polystyrene beads, magnetic beads, nitrocellulose strips, membranes, microparticles such as latex particles, chips of glass, plastic, derivatized plastic, metal and silicon, and others.

According to a first embodiment of this invention, a test sample which may contain HCV IgM is contacted with a solid support to which HCV antigen has been attached, to form a mixture. This mixture is incubated for a time and under conditions sufficient to form HCV antigen/antibody complexes. Then, a probe comprising a mammalian anti-human IgM to which an enhancer has been attached is contacted with the HCV antigen/antibody complexes, to form a second mixture This second mixture is incubated for a time and under conditions sufficient to form a second mixture reaction product. Next, an indicator reagent which comprises an enhancer compound binding member and a signal generating compound capable of generating a measurable signal is contacted with the second mixture reaction product . This third mixture is incubated for a time and under conditions sufficient to form indicator reagent reaction products. The presence and/or amount of HCV IgM is determined by detecting the signal generated. The amount of HCV IgM present in the test sample is proportional to the signal generated.

Another embodiment of the invention comprises an assay wherein a test sample which may contain HCV IgM is contacted with a solid support to which HCV antigen has been attached, to form a mixture. This mixture is incubated for a time and under conditions sufficient to form HCV antigen/antibody complexes. Then, an indicator reagent which comprises a signal generating compound capable of generating a measurable signal attached to a specific binding member for HCV IgM is contacted with the complexes, to form a second mixture. This second mixture is incubated for a time and under conditions sufficient to form a reaction. The presence and/or amount of HCV IgM present in the test sample is determined by detecting the signal generated. The amount of HCV IgM present in the test sample is proportional to the signal generated.

In yet another assay configuration, a test sample is contacted with mammalian anti-human IgM which is coated on a solid phase, and reacted for a time and under conditions sufficient for human IgM/anti-human IgM complexes to form. These complexes then are contacted with a probe which comprises at least one HCV antigen selected from HCV CORE, HCV 33c and HCV c-100 attached to an enhancer compound. The preferred enhancer compound is biotin. These are reacted for a time and under conditions sufficient to form antigen/antibody/antibody complexes. Next, these complexes are contacted with an indicator reagent comprising a signal generating compound conjugated to an enhancer compound binding member. The most preferred signal generating compound is the enzyme alkaline phosphatase. The most preferred enhancer compound binding member is anti-biotin. The resultant mixture is reacted for a time and under conditions sufficient for a reaction to occur. If an enzyme is utilized, the signal is detected and measured after addition of an enzyme substrate. The amount of HCV IgM present in the test sample is proportional to the signal generated.

Yet other assay configurations can be adapted to detect HCV IgM by practicing the teachings of this invention, and are contemplated to be within the scope of this invention.

It is preferred that the test sample be treated such as to remove rheumatoid factor-like substances which may be present in the test sample and which may interfere with the performance of the assay. Such treatment can be performed in a variety of ways known to those skilled in the art and include preadsorbing the test sample with protein A or protein G, heat aggregated IgG, and the subjection of the test sample to an amount of anti-human IgG sufficient to bind a substantial amount of the interfering rheumatoid factor-like substances. The most preferred method for treating the test sample comprises diluting the test sample in a diluent sample buffer which contains an amount of goat anti-human IgG sufficient to bind the rheumatoid factor-like substances which may be present in the test sample. This dilution step preferably is performed prior to contacting the test sample with the capture reagent, HCV antigen. The preferred buffer is one which can remove any interfering IgG which may be present in the test sample. Thus, buffers which contain a sufficient quantity of anti-IgG can be used as the diluent sample buffer. Examples of buffers that can be used in the assay include Tris buffered saline, phosphate buffered saline, and others known to those skilled in the art. The most preferred buffer comprises a Tris buffered saline (pH 7.2) to which goat anti-human IgG has been added. Further, other compounds may be added to this buffer to block non-specific binding. The selection of these compounds depends upon the constituents chosen for the assay, and are within the ordinary skill of the artisan.

The origin of the mammalian anti-human IgM may be goat, rabbit, sheep, or other mammalian anti-human IgM known in the art. Preferably, the mammalian origin of the anti-human IgM is goat.

When HCV antigen is used as a capture reagent in the assays described herein, at least one HCV antigen is used, either when attached to a solid phase or in solution. These antigens include HCV CORE, HCV 33c and HCV c-100. We have determined that HCV CORE is the most preferred antigen to utilize in performing the assay of the invention, but that HCV 33c and HCV c-100 also can be used alone or in any combination. Thus, HCV CORE antigen can be combined with HCV 33c and/or c-100, or other HCV antigens, and used as capture antigens in methods described herein.

It also is contemplated that a sandwich assay can be performed wherein a soluble capture reagent can include an analyte-specific binding member which has been bound to a charged substance such as an anionic substance. The present invention also can be used to conduct a competitive assay. In a competitive configuration, the soluble capture reagent again includes a specific binding member which has been attached to a charged substance, such as an anionic polymer, with which to bind a specific binding partner. Assays which utilize such charged substances are described in pending patent application U. S. Serial No. 150,278 filed January 29, 1988 and U.S. Serial No. 375,029 filed July 7, 1989, which both enjoy common ownership and both of which are incorporated herein by reference.

Alternatively, it also is contemplated that the assay can be performed by scanning probe microscopy, in which an analyte, analyte analog or analyte specific substance which has been bound to a test piece, is contacted with the test sample suspected of containing the analyte, incubated for a time and under conditions sufficient for a reaction to occur, and then the presence of analyte is determined by using scanning probe microscopy. Such an assay is the subject matter described in pending U. S. Patent Application Serial No. _____, (Attorney Docket D-17808) filed February 28, 1991, which enjoys common ownership and is incorporated herein by reference.

The present invention will now be described by way of Examples, which are intended to demonstrate, but not to limit, the spirit and scope of the invention.

EXAMPLES

Example 1

Semi-automated Dot-Blot Immunoassay

The ABBOTT MATRIX™ analyzer (available from Abbott Laboratories, Abbott Park, IL) was used to detect an array of purified recombinant HCV antigens coated on a test card comprising nitrocellulose in a semi-automatic dot-blot immunoassay. This technology has been described by co-owned and co-pending patent applications U. S. Serial Nos. 227,408 (published as European Patent

Office [EPO] Publication 0 353 591), 227,590 (published as EPO Publication 0 353 592), 227,586 (published as EPO Publication 0 353 589) and 227,272 (published as EPO Publication 0 353 590), each of which is incorporated herein by reference. Briefly, the test cards were prepared as detailed hereinbelow, and the so-prepared test cards were used for assay as described herein. Each test card contained a negative control. The negative control allowed for a self-blanking and validity check.

An HCV test card was developed that consisted of the HCV clone c100-3 (as described by Kuo et al., Science 244:362-364 [1989]) chimeric polypeptide expressed in yeast plus recombinant HCV polypeptides expressed in E. coli that included those from pHCV-23 (c100 fragment, lacking the first 107 N-terminal amino acids), pHCV-29 (CKS-33c), pHCV-34 (CKS-CORE) and pHCV-35 (λ pL CORE), and pHCV-45 (NS4/NS5 junction). FIG. 1 is a map of the rDNA expressed regions of HCV which are described herein. The amino acids sequences of these HCV polypeptides are known in the art and are described in European Patent Application 0 388 232, published September 19, 1990 (see pages 32 and 34)., which is incorporated herein by reference.

All proteins were expressed as CMP-KDO synthetase (CKS) fusion proteins (as taught by T. J. Bolling and W. Mandecki, "An Escherichia coli expression vector for high-level production of heterologous proteins in fusion with CMP-KDO synthetase," Biotechniques 8:488-490 [1990]), with the exception of pHCV-35, which was expressed in a lambda (λ) pL expression system. In addition, each test card contained a reference spot (negative control) to detect background signal which also proved useful in verifying the assay. The preparation of the recombinant polypeptides were individually optimized for spotting by adsorption onto the solid support which comprised nitrocellulose. The preferred buffers, pH conditions and spotting concentrations were as summarized in Table 1 which were as described in co-pending and co-owned patent application Serial No. 532,489, which is incorporated herein by reference. However, it was discovered that in addition to these optimal factors, successful applications of the polypeptides to the test card also were accomplished at different pH values, detergent compositions and salt concentrations.

TABLE 1

Conditions for Application of HCV Polypeptide

PLASMID/ PROTEIN	ng/SPOT	SPOTTING BUFFER
c100	100-150	20 mM Tris-HCl, 0.9% NaCl, 0.015% SDS, pH 8.3
pHCV-23/CKS-BCD	100-150	20 mM Tris-HCl, 0.9% NaCl, 0.015% SDS, pH 8.3
pHCV-29/CKS-33c	100-150	50 mM Naphosphate, 0.01% Triton X-100®, pH 6.5
pHCV-35/CORE	100-150	50 mM Tris-HCl, 0.0025% Tween-20®, pH 8.5
pHCV-34/CKS-CORE	75-100	50 mM Naphosphate, 0.0025% Tween-20®, pH 12.0
pHCV-45/CKS-E	100-150	50 mM Tris-HCl, 0.015% SDS, pH 8.5

After spotting, the solid support was dried at ambient temperatures, after which time the solid support was rinsed with Tris-buffered saline (TBS, which contained 20 mM Tris buffer, 0.5 M NaCl, 0.1% NaN_3, at pH 7.4 to 7.6). The solid support then was overcoated with a solution of (1%) porcine gelatin, (1%) casein acid hydrolysate and (5%) Tween-20® in TBS for approximately 30 minutes at 35° C. The solid support was rinsed an additional two times with TBS, and then it was air-dried in an oven at 37° C before assembly into a test cell cartridge.

Example 2

HCV Assay Procedure for Determination of IgM

Ten (10) µl of test sample was mixed with one (1) ml of a specimen diluent (which consisted of 1% w/v bovine serum albumin [BSA], 0.5% w/v non-fat dry milk, 0.03% yeast extract and 5% v/v E. coli lysate containing CKS protein in 20 mM TBS which contained 1.5% v/v Brij-35®, 0.1% w/v EDTA, and 0.1% NaN_3). If the diluent contained mammalian anti-human IgG for controlling rheumatoid factor-like interferences, then the sample/diluent mixture was allowed to incubate at room temperature for approximately 15 minutes. Following this incubation, the sample/diluent mixture was centrifuged at 12,000 rpms for five minutes for removal of precipitated IgG aggregates. The supernatant was used in the assay as the diluted test sample. One (1) ml of the diluted test sample was transferred to a test cell prepared as described hereinabove. The test cells were incubated with test samples for approximately 60 minutes at 35° C.

Then, the test cell was washed with TBS for approximately 15 minutes in the ABBOTT MATRIX™ analyzer. The wash comprised a series of wash steps which washed and rinsed the test cell at least three times. Next, one (1) ml of anti-human IgM:biotin probe was diluted into a probe diluent (ABBOTT MATRIX™ probe diluent, commercially available from Abbott Laboratories, Abbott Park, IL) at 200 ng/ml. The most preferred anti-human IgM was goat anti-human IgM (goat anti-human IgM $F(ab')_2$:biotin conjugate, available from Sigma Chemical Co., St. Louis, MO), although it also was determined that the whole goat anti-human IgM:biotin conjugate probe may be utilized. This mixture was incubated for 30 minutes at 35°C. Following this incubation, another 15 minute wash was performed as detailed hereinabove. Then, one (1) ml of an anti-biotin:alkaline phosphatase conjugate was added to the reaction cell and incubated for 30 minutes at 35°C. This anti-biotin antibody utilized was a rabbit polyclonal antibody. The enzyme was an alkaline phosphatase derived from calf intestine (Boehringer Mannheim, Indianapolis, IN) or E. coli recombinant alkaline phosphatase (Abbott Laboratories, Abbott Park, IL). Following this incubation, another 15 minute wash was performed as detailed hereinabove. Next, one (1) ml of 5-bromo-4-chloro-3-indole phosphate (BCIP) was added to the reaction cell and this mixture was incubated for 30 minutes at 35°C. Following this incubation, a final 15 minute wash was performed as described hereinabove.

After completion of the final wash, the ABBOTT MATRIX™ analyzer dried the reaction cell (at 35°C for 25 minutes) and determined the reflectance at defined locations within the array of the reaction cell, thereby providing an objective measurement of the extent of the individual reactions. The results appeared as DR (reflectance density) or ΔDR (a self-blanked result in which the DR value for a negative control spot was subtracted from the test spot DR). Reactivity cutoff values were calculated for each antigen based on selected populations, as defined hereinbelow.

Example 3

Selected Populations

In order to qualify the anti-HCV-IgM assay, characterized populations of both negative and positive specimens were required. It was

decided that the negative population group would be demonstrated by requiring all specimens to be anti-HCV negative by current HCV assays, preferably those which utilized recombinant HCV proteins. The positive population was harder to define, since none had been documented to date. It was decided to target high risk or high probability populations, such as anti-HCV reactive populations and populations reactive for surrogate markers, such as alanine aminotransferase (ALT). These three groups were (1) negative population used for establishing an assay cutoff, (2) a surrogate marker reactive population, namely elevated ALT samples and (3) an anti-HCV reactive population, namely the "Sacramento specimens."

1. Determination of Assay Cutoffs with A Negative Population

A negative control was used to blank each reaction cell. The reaction cells were blanked by subtracting the negative control DR value from the DR value obtained for every test spot on the reaction cell. The negative control not only blanked out sample-to-sample background inconsistencies, but it also was used as an absolute standard for assessing the amount of background present. If the negative control DR value for a particular sample exceeded a predetermined DR value, the validity of the test results were considered to be in doubt and the assay results were invalidated. This dual feature of the negative control, namely self-blanking and validity check, gave the assay increased specificity control over conventional EIAs.

The method used to establish an assay cutoff for the HCV IgM immunoassay of the invention was as follows. Since the assay configuration consisted of reacting a patient specimen with multiple antigen and control spots, multiple cutoffs were determined, one for each test spot. To determine these cutoffs, a selected population of known HCV nonreactive specimens was tested by the HCV IgM assay of the invention performed by the ABBOTT MATRIX™ analyzer, as follows. Negative specimens were assayed as described herein, the ΔDR values were calculated for each antigen test spot for each sample, and statistics were performed on the population set. The mean and standard deviation were calculated over the entire data set for the negative control spot DR values and for each antigen spot ΔDR values. The cutoff was generally set at a

value of the antigen spot mean ΔDR value + 6 standard deviations (sd). In general, it was determined that the cutoff values should be in the neighborhood of ΔDR = 0.030-0.050, with a maximum valid negative control DR value = 0.100-0.150. Once the basic cutoff values were established, the calculation of each specimen's reactivity was easily determined. One additional factor which was considered was an assay offset due to the nonlinearity of reflection reads. It was determined that the cutoff for each spot must be adjusted for each ABBOTT MATRIX™ reaction cell based on the value of the negative control spot. The calculation was

$$\text{cutoff}(i) = \text{cutoff}(0) + 0.15 * \text{Negative Control DR}(i)$$

where cutoff(i) was the cutoff to be used for a specific antigen on the test cell assayed with sample i, cutoff(0) was the calculated cutoff (mean + 6 sd) and Negative Control DR(i) is the Negative Control DR value for sample i. Regardless of the cutoff value calculated, the minimum cutoff value was
ΔDR = 0.030.

2 Elevated ALT samples

A population of 91 specimen plasma units with elevated ALT values was sourced and tested by the HCV IgM assay of the invention in order to identify HCV IgM reactive specimens. These specimens previously had been screened for anti-HCV (IgG) activity by several HCV assays: a prototype assay ABBOTT MATRIX™ HCV assay; a prototype assay ABBOTT IM_X® HCV assay; a commercially available, licensed assay designated as the ABBOTT "1.0" assay which employs HCV c100 as the capture antigen; and a prototype assay ABBOTT "2.0 EIA" which utilizes two additional recombinant HCV proteins (33c and CORE) (all the these tests are available from Abbott Laboratories, Abbott Park, IL). Of the 91 specimens assayed, three (3) specimens (specimen numbers 1010, 1034 and 1089) were HCV IgG reactive in these various HCV assays (ABBOTT MATRIX™ HCV assay, the ABBOTT "1.0" assay, and the prototype ABBOTT "2.0 EIA"). These three samples were tested by the HCV IgM assay of the invention following the procedure detailed hereinabove in Examples 1 and 2. One of these three specimens, specimen 1010, was consistently reactive in the HCV IgM assay of the invention. This specimen was subjected to several confirmation

procedures as detailed hereinbelow and was chosen as a positive control for the HCV IgM assay. Another sample, sample number 1089, showed cell lot-to-lot reactive/nonreactive behavior. This reactivity was determined to be false reactivity, neutralizable by the addition of anti-human IgG to the specimen diluent.

3. Anti-HCV Reactive Populations

Another population group from which to pursue HCV IgM reactive specimens was identified as a collection of specimens referred to as the "Sacramento specimens." These specimens were obtained from blood screening services in Sacramento, CA (Sacramento Medical Foundation, Center for Blood Research) and represented all Ortho HCV (1.0) EIA repeat reactive specimens from the first 20,000 blood units screened at these blood screening services in Sacramento, CA after the availability of the Ortho-distributed HCV assay (commercially available as Ortho™ HCV ELISA Test System, Ortho Diagnostics, Raritan, NJ).

Sixty-four (64) of 142 specimens shown to be repeatedly reactive by the Ortho anti-HCV Assay (Ortho™ HCV ELISA Test System, Ortho Diagnostics, Raritan, NJ) were shown to have IgG reactivity to HCV CORE, HCV 33c, and HCV c-100 by the prototype assays described herein for HCV (ABBOTT MATRIX ™ HCV IgG AND ABBOTT HCV "2.0 EIA").

It was observed that the HCV IgM assay had distinctly different results depending upon the composition of the specimen diluent. This was determined by experiments which were run either with ABBOTT MATRIX™ HCV (IgG) specimen diluent, or with mammalian anti-human IgG added to ABBOTT MATRIX™ HCV (IgG) specimen diluent. Mammalian anti-human IgG was added to the specimen diluent in order to precipitate out rheumatoid factor-like substances, if they were present in the test sample. Mammalian anti-human IgG was added to the test sample at the concentration indicated in Example 2, and the test samples were assayed as described in Examples 1 and 2. These two diluents yielded the following results. It was found that 36 (56.2%) of 64 ABBOTT MATRIX™ HCV reactive specimens were reactive for HCV IgM by the assay of the invention by using the ABBOTT MATRIX™ analyzer with the first diluent, whereas seven (7) (24.1%) of 29 test samples were

reactive for HCV IgM by the assay of the invention when using the diluent which also contained anti-human IgG while using the ABBOTT MATRIX™ analyzer. Hence, it was determined that 22 of 29 reactive specimens displayed false IgM reactivity when the first diluent was used. All seven (7) of the "true" HCV IgM reactive samples, i.e., those reactive with the modified diluent, exhibited reactivity to the HCV CORE protein (CKS-CORE). In addition, one sample showed reactivity to HCV CORE and HCV 33c, while another sample showed reactivity to all three antigens. These data are summarized in Table 6.

Example 4

Serial Bleeds From Post-Transfusion (PT) NANBH Patients

Once a prototype assay for anti-HCV IgM was established, the next step was to identify which antigens elicited an HCV IgM response and to correlate such HCV IgM responses with the time dependent serological profiles of both acute and chronic phase patients. Serial bleed samples from infected patients, drawn at regular time intervals throughout the acute phase of the disease (before and during the presentation of symptoms) and through the post-acute phase (resolved or chronic infection), were used to assess the efficacy of potential acute phase markers.

Three distinct serial bleed specimen groups were evaluated for IgM response to HCV SOD-C100, CKS-33c and CKS-CORE recombinant antigens. These groups included a seroconversion panel (unit volume quantities) and serial bleed specimens obtained from NIH.

1. Seroconversion Panel

The Seroconversion Panel consisted of a 19 member panel of serial bleeds from three (3) donors. Each of these donors was seronegative for all HCV markers in the first serial bleed. During the time period over which these bleeds were obtained, each patient seroconverted to reactive for HCV antibody (IgG) to one or more HCV antigens. Panel members 1-6 are from patient 1, 7-11 from patient 2 and 12-17 from patient 3.

This panel was tested by Abbott HCV 1.0 EIA, 2.0 EIA, ABBOTT MATRIX™ HCV assays (available from Abbott Laboratories, Abbott Park, IL) and the HCV IgM assay of the invention (as detailed in

Examples 1 and 2). The results for these assays are summarized in Table 2. The data from Table 2 clearly discriminates the difference between the 1.0 EIA (SOD-c-100 only) results and the 2.0 EIA (SOD-c-100, CKS-33c and CKS-CORE). It can be seen from the results in Table 2 that HCV CORE and 33c reactivity frequently preceded c-100 reactivity. For this particular group of patients' serial bleeds, the HCV IgM assay showed reactivity one bleed before the prototype ABBOTT MATRIX™ HCV (IgG) assay in one case, and one bleed later in another. In general, the HCV IgM response was transient, showing itself early in the infection, peaking quickly and then dropping.

TABLE 2

Reactivity of Seroconversion Panel

Panel	ALT	Bleed	1.0	2.0	HCV IgG†			HCV IgM†		
Member	IU/L	(Days)	EIA	EIA	c100	33c	CORE	c100	33c	CORE
1	41	0	-	-	0.0	0.0	0.1	0.2	0.5	1.0
2	51	7	-	-	0.1	0.0	0.1	0.3	0.1	0.1
3	63	14	-	-	0.1	0.1	0.1	0.1	0.0	0.0
4	183	24	-	-	0.1	0.7	47.4*	0.1	0.1	69.9*
5	44	44	+	+	2.6*	322.1*	202.1*	0.4	0.3	100.8*
6	72	72	+	+	119.8*	417.7*	204.0*	0.5	2.1	94.3*
7	27	0	-	-	0.1	0.0	0.1	0.0	0.0	0.0
8	180	11	-	-	0.0	0.1	0.4	0.0	0.0	6.4*
9	401	40	-	+	0.3	372.8*	146.5*	0.0	0.0	91.8*
10	na	82	+	+	124.9*	415.1*	157.1*	0.0	0.0	13.9*
11	na	95	+	+	194.8*	574.8*	241.4*	0.0	0.1	5.5*
12	14	0	-	-	0.1	0.0	0.1	0.0	0.0	0.0
13	17	7	-	-	0.1	0.1	0.1	0.0	0.0	0.0
14		10	-	-				0.0	0.0	0.0
15		14	-	-				0.0	0.0	0.0
16	274	24	-	-	0.1	0.4	0.4	0.0	0.0	0.0
17	346	28	-	-	0.1	15.2*	0.4	0.0	0.0	0.1
18	1175	44	+	+	7.2*	364.5*	91.4*	0.5	0.4	4.3*
19	429	72	+	+	13.2*	310.9*	95.0*	0.0	0.1	1.3*

†S/CO values are reported; S/CO ≥ 1.00 is considered reactive; * these samples were considered reactive.

The panel was tested for HCV IgM antibodies using both a diluent without mammalian anti-human IgG and the same composition of diluent which contained mammalian anti-human IgG according to the procedures detailed in Examples 1 and 2. The data are presented in Table 3. It can be seen by the data presented in Table 3 that the addition of the anti-human IgG to the diluent had no diminishing effect on the reactivity of the assay. Some small increase in reactivity may have been present for the later reactive bleeds due to the removal of anti-HCV IgG in the specimen through precipitation with the anti-human IgG. High titer anti-HCV IgG antibody competed with the anti-HCV IgM for binding to the solid phase antigen, thus reducing the amount of IgM bound, which resulted in a reduction of the reactive signal. The removal of the IgG from the sample by precipitation with anti-human IgG eliminated the observed competition, which allowed all available IgM to bind to the solid phase antigens and provided maximal reactive signal.

TABLE 3

Comparison of HCV IgM Assay Specimen Diluents

Panel Member	Without anti-human IgG†			With anti-human IgG†		
	c-100	33c	CORE	c-100	33c	CORE
1	0.27	0.00	0.43	0.20	0.47	1.00
2	0.30	0.07	0.00	0.33	0.10	0.10
3	0.23	0.03	0.10	0.07	0.00	0.00
4	0.27	0.03	63.77*	0.07	0.10	69.93*
5	1.00	0.00	89.90*	0.40	0.27	100.83*
6	0.70	0.00	51.80*	0.50	2.13	94.30*
7	0.00	0.00	0.00	0.00	0.00	0.00
8	0.00	0.00	9.23*	0.00	0.00	6.43*
9	0.07	0.20	94.03*	0.00	0.00	91.83*
10	0.07	0.00	21.23*	0.00	0.00	13.87*
11	0.07	0.13	2.83*	0.00	0.13	5.53*
12	0.07	0.00	0.00	0.00	0.00	0.00
13	0.07	0.03	0.00	0.00	0.00	0.00

14	0.07	0.03	0.07	0.00	0.00	0.00
15	0.13	0.03	0.00	0.00	0.00	0.00
16	0.10	0.03	0.07	0.00	0.00	0.00
17	0.10	0.07	0.03	0.03	0.00	0.10
18	1.30*	0.63*	5.07*	0.47	0.3[illegible]	4.30*
19	0.23	0.20	0.63	0.00	0.07	1.30*

† S/CO ≥ 1.00 is considered reactive; * indicates a reactive sample.

2. NIH Post-transfusion serial bleeds

A well-documented study of 19 post-transfusion non-A, non-B hepatitis patients by H. Alter et al. (supra) detailed the time course of antibody response to HCV c-100 antigen. As a part of an Abbott collaboration with NIH, the serial bleeds from these patients were studied by using the ABBOTT MATRIX™ HCV IgG assay, and the IgM assay of the invention. These serial bleed specimens covered the acute phase of the disease and thereby provided a valuable resource for identifying acute phase markers. Some patients were followed for a prolonged period over ten years.

All 19 patients in the study had developed chronic NANBH. Of the 19 patients tested, 17 (89.5%) showed HCV IgM response of varying types, while two (10.5%) patients did not show detectable levels of HCV IgM antibody. Nine of 17 patients showing IgM reactivity (52.9%) demonstrated acute phase transient HCV CORE IgM response, ranging from 28 to 135 days in duration, that either preceded or was concomitant with the onset of active HCV CORE IgG antibody production. Four (4) of 19 patients showed later CORE IgM reactivity.

Two (2) of 17 patients (11.7%) had acute 33c IgM, while only one patient showed acute phase c-100 IgM antibody. Four patients exhibited 33c IgM antibody after detection of 33c IgG antibody.

Example 5

Effect of Rheumatoid Factor-Like Substances On Determination of IgM

Tests were conducted to determine the effect of rheumatoid factor-like substances, if any, on the results obtained for HCV IgM on test samples. The tests were conducted as follows. Test specimens were

"spiked" with rheumatoid factor at varying concentrations to sample volume of 10 µl. Specimen No. 170 was strongly reactive for anti-CORE, 33c and c-100, but negative for HCV IgM regardless of pretreatment with anti-human IgG. Table 4 and FIG. 2 show data obtained on specimen number 170 when tested for HCV antigens c-100, "BCD", 33c, and CORE. Then, assays were performed to determine the amount, if any, of HCV IgM antibody to these HCV antigens by following the methods as described in Examples 1 and 2.

TABLE 4

Effect of Rheumatoid Factor-Like Substances on IgM HCV Assay†

Vol.	RF IU/ml	C100	BCD	33c	Avg.	CORE	AVG.
10	0	0.000Δ	0.000Δ	0.000Δ	0.000Δ	0.000Δ	0.000Δ
10	0	0.000	0.001	0.000		0.000	
10	333	0.001	0.000	11.030	10.422	5.660	5.050
10	333	0.001	0.000	9.405		4.448	
10	571	0.003	0.000	10.105	9.405	3.235	3.604
10	571	0.004	0.000	8.705		3.973	
10	666	0.008	0.000	10.164	9.868	3.693	3.426
10	666	0.009	0.000	9.572		3.156	
10	750	0.004	0.000	8.451	9.198	1.582	2.160
10	750	0.004	0.000	9.855		2.739	
10	888	0.021	0.013	8.396	8.725	0.362	0.348
10	888	0.010	0.000	9.054		0.334	
10	-	0.022	0.000	0.000		0.000	0.000
10	-	0.004	0.000	0.000		0.000	

† The assay was conducted without addition of anti-IgG.

Δ These values represent DR readings. Values greater than 0.06 are considered reactive.

The data from the Table and FIG. 2 demonstrate that Rheumatoid Factor produced false positive HCV IgM reactivity in a sample that was negative for IgM but positive for IgG. The degree of reactivity was dependent upon the amount of RF present. The relative false positive reactions towards 33c and CORE was not correlated to or proportional to each other.

The neutralization of RF-induced false reactivity in the HCV IgM assay of the invention was assessed, as well as the proper titer of anti-human IgG activity for use in the assay, as follows. False reactive samples were created by combining a specimen strongly reactive for HCV IgG and nonreactive for HCV IgM (10µl) with a high titer RF specimen (at 2, 5 or 10 µl) as previously described. The specimen diluent was spiked with varying amount of anti-human IgG antisera (from 0 to 100 µl per ml of diluent). Assay for HCV IgM of the false reactive specimens together with negative and positive controls was conducted following the methods described in Examples 1 and 2.

The results are summarized in Table 5. Anti-human IgG was shown to neutralize the false reactivity of the prepared false positive sample. As the titer of anti-human IgG antisera increased in the specimen diluent, the percentage of neutralization increased. At approximately 40 µl of antisera per ml of diluent, the neutralization was complete and larger amounts of antisera did not improve the neutralizing performance. Thus, 40 µl of antisera per ml was chosen as the appropriate titer for neutralizing specimens when diluted.

TABLE 5

Effect of Anti-human IgG on RF-induced False IgM Reactive Samples

HCV IgG Specimen Volume	RF Spec. Vol.	Anti-human IgG Volume	anti-HCV 33c ΔDR	anti-HCV 33c %Neut.	anti-HCV CORE ΔDR	anti-HCV CORE %Neut.
10 µl	2 µl	0 µl	9.844		6.41	
10	2	20	1.388	85.9%	0.310	95.3%
10	2	40	0.877	91.0%	0.005	99.9%
10	2	60	0.040	99.5%	0.001	99.9%
10	5	0	9.259		7.905	
10	5	20	3.050	67.0%	0.819	89.6%
10	5	40	3.656	60.5%	0.273	96.5%
10	5	60	0.094	98.9%	0.019	99.7%
10	10	0	7.546		6.313	
10	10	20	3.077	59.2%	0.981	84.5%
10	10	40	1.230	83.7%	0.149	97.6%

10	10	60	0.411	94.6%	0.000	100.0%
10	10	70	0.069	99.1%	0.000	100.0%
10	10	100	0.042	99.4%	0.000	100.0%

Tests were conducted to determine the effect, if any, of the presence of anti-human IgG in the test sample on the determination of HCV IgM in that test system. Accordingly, test samples were obtained from the Sacramento Medical Foundation ("Sacramento samples"). The effect of the presence or absence of goat anti-human IgG was assayed to determine whether the results obtained for the test samples was due to the presence of IgG in the test sample, which cross-reacted with IgM, or due to the actual presence of IgM. Each sample was tested for IgM in side-by-side comparison tests by the assay of the invention following the methods of Examples 1 and 2, in which each sample was divided into two aliquots. One aliquot was treated with goat anti-human IgG ("treated aliquot") and the other aliquot was not treated with goat anti-human IgG ("untreated aliquot"). Test sample aliquots were processed and tested according to Examples 1 and 2. The data are presented in the following Table 6.

TABLE 6

Sacramento Samples (IgM Positive) With and Without anti-human IgG

	WITHOUT GOAT ANTI-HUMAN IgGΔ				WITH GOAT ANTI-HUMAN IgGΔ			
SAMPLE	NEG. C.	c-100	CORE	33c	NEG. C	c-100	CORE	33c
1010†	0.023	0.148*	1.340*	0.023	0.019	0.190*	1.037*	0.030
39	0.019	0.003	1.943*	0.000	0.012	0.000	0.000	0.000
43	0.043	0.018	3.283*	0.046	0.030	0.013	1.133*	0.107
45	0.067	0.012	1.844*	0.072	0.055	0.009	0.029	0.000
47	0.034	0.043	0.077*	0.003	0.070	0.111*	0.205*	0.058
56	0.007	0.000	0.052*	0.000	0.005	0.000	0.000	0.000
57	0.049	0.009	0.022	0.000	0.034	0.017	0.002	0.009
58	0.007	0.000	0.070*	0.000	0.006	0.000	0.000	0.000
76	0.036	0.017	0.913*	0.004	0.024	0.008	0.529*	0.002

	78	0.008	0.006	0.133*	0.009	0.005	0.000	0.000	0.000
	85	0.007	0.003	1.030*	0.004	0.005	0.000	0.086*	0.000
	89	0.009	0.002	2.601*	0.000	0.008	0.001	0.251*	0.000
	91	0.006	0.000	0.343*	0.504	0.003	0.000	0.000	0.000
	97	0.006	0.000	6.095*	0.002	0.005	0.006	0.000	0.000
	105	0.116	0.082	0.389*	0.066	0.109	0.030	0.017	0.014
	116	0.015	0.018	9.398*	3.580	0.009	0.002	0.002	0.003
	153	0.017	0.003	4.683*	0.005	0.008	0.001	0.015	0.002
	159	0.004	0.000	7.362*	0.000	0.004	0.000	0.000	0.000
	163	0.021	0.005	1.224*	0.031	0.011	0.002	0.003	0.000
	171	0.027	0.012	5.538*	0.128*	0.013	0.003	0.003	0.001
	172	0.005	0.000	3.403*	0.022	0.003	0.000	0.001	0.000
	174	0.016	0.005	0.019	0.005	0.010	0.003	0.005	0.001
	179	0.008	0.001	0.134*	0.005	0.005	0.000	0.000	0.000
	180	0.018	0.005	2.881*	0.000	0.013	0.002	2.499*	0.000
	181	0.011	0.000	3.449*	2.343	0.007	0.000	0.000	0.000
	186	0.113	0.023	0.198*	0.021	0.058	0.025	0.000	0.000
	187	0.006	0.000	2.286*	0.000	0.004	0.000	0.001	0.000
	190	0.102	0.035	8.117*	0.001	0.071	0.020	0.008	0.000
	196	0.015	0.004	1.093*	0.299*	0.007	0.001	0.000	0.000
	203	0.020	0.008	0.008	0.0[illegible]	0.012	0.002	0.003	0.000
	208	0.011	0.086	5.552*	1.535*	0.006	0.002	0.005	0.002
	209	0.016	0.004	0.133*	0.002	0.009	0.002	0.004	0.000
	210	0.009	0.003	0.114*	0.004	0.005	0.000	0.001	0.000
	165	0.007	0.004	0.353*	0.002	0.005	0.001	0.162*	0.000

Δ All values are DR readings.

† Sample 1010 was used as the positive control.

* Indicates a "positive" (reactive) reaction.

The data of Table 6 demonstrate that specimens that tested "positive" reactive without the inclusion of anti-IgG in the specimen diluent did not necessarily test positive when anti-human IgG was added to the specimen diluent; only seven remained positive after adsorption with anti-human IgG. The data demonstrates that a false positive reaction for HCV IgM can occur if the test specimens are not treated to remove the interference of rheumatoid factor-like substances.

Example 6
Confirmation Studies

The reactivity of specimens to the anti-IgM label in the assay format of the invention did not in itself establish that IgM class anti-HCV antibodies in the sample were responsible for the assay response. In order to confirm the presence of HCV specific IgM reactivity, several studies were performed, namely, fractionating the sample on a molecular weight sizing column, treating the sample with anti-IgG (human) to neutralize RF-like interference and treating the sample with dithiothreitol (DTT) which destroyed the IgM reactivity but not the reactivity of IgG.

1. Sizing Profiles Over Sephacryl S-300 Columns

Several samples were selected for size exclusion fractionation over an S-300 chromatography column. The purpose of fractionating these specimens was to generate HCV results profiles using either anti-IgM or anti-IgG. It was determined that IgM and IgG should resolve into different fractions by molecular size; hence, anti-IgM and anti-IgG reactivity should occur for different fractions. Seven anti-HCV IgM specimens reactive by the method of the invention were sized on an S-300 column (Sephacryl S-300 Column, available from Pharmacia, Piscataway, NY). This group included six (6) samples from the Sacramento study group [6/8 reactive after treatment with anti-human IgG Antibody (Ab) blocker (potential true positives), 2/8 neutralized with Ab blocker (false positives)] and one elevated ALT sample (sample 1010). Data from one sample 1010 is presented hereinbelow in Tables 7 and 8 and FIGS. 3 and 4.

TABLE 7
Probe-IgM $(FAB')_2\Delta$

Sample	Neg. C.	CORE	33c	c-100
1010	0.045	1.803	0.059	0.377
Fraction No.:				
14	0.003	0.003	0.003	0.003
17	0.010	0.539	0.016	0.003
19	0.042	2.723	0.126	0.600
21	0.021	1.142	0.042	0.175

23	0.003	0.293	0.011	0.023
25	0.004	0.020	0.005	0.006
27	0.003	0.006	0.003	0.004
31	0.003	0.003	0.003	0.003
39	0.005	0.004	0.004	0.003

Δ These values represent DR readings. Values greater than 0.06 are considered positive.

TABLE 8

Probe-IgG (FAB')2Δ

Sample	Neg. C.	CORE	33c	c-100
1010	0.028	6.511	8.493	0.096
Fraction No.:				
14	0.004	0.010	0.004	0.004
17	0.004	0.012	0.004	0.004
19	0.005	0.026	0.007	0.005
21	0.006	0.318	0.043	0.007
23	0.010	2.539	3.230	0.022
25	0.020	6.383	8.572	0.078
27	0.018	6.004	7.277	0.068
31	0.006	0.791	0.258	0.008
39	0.004	0.009	0.007	0.006

Δ These values represent DR readings. Values greater than 0.06 are considered positive.

Antibody activity in the majority of the fractions from this sample was measured with IgG probe as well as IgM probe. As the tables show, fractions 17-21 exhibited strong IgM activity, while fractions 23-27 exhibited strong IgG activity. Fractions showing IgM activity were eluted in the void volume which corresponded to the molecular weight of IgM antibody. These fractions had very low or negligible amounts of IgG antibody. Fractions showing IgG activity were eluted later (after the void volume) and had no IgM activity.

A 0.7 X 50 cm column was packed with Pharmacia S-300 gel. One (1) ml of sample was filtered (0.2 μm filter, available from Millipore,

Bedford, MA) and loaded on the column. Elution with 0.05 M Tris, pH 7.5 was run at a rate of 6-10 ml per hour. Half ml fractions (0.5 ml) were collected. Fractions were measured for absorbance at 280 nm and either 340 or 400 nm.

The HCV IgM assay of the invention was run as detailed in Examples 1 and 2 using 10 µl of undiluted specimen or 25 µl of column fractionated specimen diluted into 1 ml of specimen diluent. The anti-IgM and anti-IgG specific probes were goat anti-human $(Fab')_2$:biotin conjugates (Sigma Chemical Co., St. Louis, MO) run at 200ng/ml. The enzyme reagent was an anti-biotin:*E. coli* alkaline phosphatase conjugate. The enzyme substrate was BCIP. The data are presented in Table 9.

TABLE 9

Sizing Profiles for IgM Reactive and False Reactive Specimens

ID	Fraction No.	IgM Assay (ΔDR)			IgG Assay (ΔDR)		
		c100	33c	CORE	c100	33c	CORE
43	14	0.00	0.00	0.02	0.00	0.00	0.02
	15	0.00	0.00	0.35	0.00	0.05	0.74
	25	0.00	0.00	0.00	5.07	10.11	8.42
89	17	0.00	0.00	0.41	0.01	0.00	0.08
	28	0.00	0.0	0.00	5.03	5.84	7.72
165	14	0.00	0.00	0.63	0.00	0.00	0.01
	24	0.00	0.00	0.00	1.07	1.34	6.85
76	18	0.01	0.00	0.96	0.00	0.00	0.04
	29	0.00	0.00	0.00	0.02	5.57	9.06
180	15	0.01	0.00	3.82	0.01	0.01	0.10
	25	0.00	0.00	0.00	1.43	7.15	.86
1010	19	0.46	0.12	2.67	0.00	0.01	0.02
	25	0.01	0.01	0.02	0.04	8.42	6.38
159	16	0.00	0.00	0.00	0.01	0.00	1.22
	26	0.00	0.00	0.00	4.58	6.45	9.12
190	26	0.00	0.00	0.00	4.58	6.45	9.12
	27	0.00	0.00	0.00	0.01	0.07	12.59

Two of the eight (8) specimens were false reactive specimens. On sizing, the peak IgM fraction had no IgM reactivity to HCV CORE, but instead had substantial IgG reactivity to CORE. This behavior appeared to be typical of false positive specimens. Also, the data was consistent with the premise that the false reactivity in these specimens was due to a rheumatoid factor-type of behavior. The aggregation of antibody due to a rheumatoid type of interaction was believed to be capable of creating a distribution of IgG-containing molecular aggregates that would be dispersed throughout the entire sizing profile. A rheumatoid factor-type of sample did not show anti-IgM reactivity for the void volume where IgM is normally found. However, IgG activity was strong not only in the IgG peak fraction region where it should be present, but also throughout the column from the void region through the IgG peak region.

Example 6

Effect of Reductants on IgM Assay

Five (5) specimens strongly reactive for anti-HCV by the Abbott anti-HCV assay (HCV IgG ASSAY, ABBOTT MATRIX ™, previously described) as well as the IgM assay of the invention were tested after treatment with a reducing agent for IgG and IgM specific reactivity to HCV antigens in the Abbott MATRIX™ assay. The IgM assay followed the methods described in Examples 1 and 2, except as follows Specifically, samples were incubated with dithiothreitol (DTT) in sodium acetate buffer at a final DTT concentration of 18 mM for 15 minutes prior to addition of specimen diluent. All five specimens gave reduction sensitive reactivity for anti-HCV CORE in the IgM assay. Reactivity to HCV CORE, HCV 33c and HCV c-100 in the IgG anti-HCV assay was not affected by the addition of a reductant to the specimen. Similarly, fractionated IgM reactivity or very low inhibition from patient 1010 was destroyed by pretreatment with DTT; however, IgG fractions of this test sample gave similar reactivity or very low inhibition in the Abbott MATRIX™ IgG assay when treated in the presence or absence of DTT. These data are shown in Tables 10 and 11.

TABLE 10

Effect of DTT Addition to Fractionated Specimen No. 1010*

Sample ID	DTT†	c-100	% Inhib.	33c	%Inhib.	CORE	%Inhib.
1010							
unfractionated	-	0.325		0.100		3.123	
IgM probe	+	0.004	98.8	0.002	98.0	0.015	99.5
IgM Fraction	-	0.380		0.127		4.212	
# 19, IgM probe	+	0.017	95.5	0.019	85.0	0.515	87.7
IgG Fraction	-	0.037		16.8		15.1	
#25, IgG probe	+	0.053		14.5	13.6	12.2	19.2

* DR readings above 0.06 is considered reactive for the assay

† "-" indicates no DTT was added to the sample; "+" indicates that DTT was added to the sample.

TABLE 11

Effect of Reducing Agent (DTT) on IgM Assay*

Sample ID	DTT†	c-100	% Inhib.	33c	%Inhib.	CORE	%Inhib.
1010	-	0.325		0.100		3.123	
	+	0.004	98.8	0.002	98.0	0.015	99.5
8130	-	2.450		7.800		14.47	
	+	0.044	97.4	0.090	99.9	0.282	98.0
6262	-	0.000		0.189		22.050	
	+	0.003		0.014	92.5	0.311	98.5
9713	-	0.022		0.111		10.221	
	+	0.013		0.016	86.0	0.247	97.5
288	-	0.008		0.033		4.845	
	+	0.002		0.014		0.05	99.0

* DR readings above 0.06 is considered reactive for the assay

† "-" indicates no DTT was added to the sample; "+" indicates that DTT was added to the sample.

Thus, the assay methods of the invention described herein can be used to assay for the presence of IgM to HCV which may be present in a

test sample. The embodiments described and presented herein are intended as examples rather than as limitations. Thus, the description of the invention is not intended to limit the invention to the particular embodiments disclosed, but it is intended to encompass all equivalents and subject matter within the spirit and scope of the invention as described and contemplated above, and as set forth in the following claims.

WHAT IS CLAIMED IS:

1. An assay for determining the presence and/or amount of IgM to Hepatitis C Virus (HCV) which may be present in a test sample, comprising:

a. subjecting the test sample to conditions sufficient to block the effect, if any, of rheumatoid factor-like substances which may be present in the test sample and contacting the test sample with HCV antigen comprising at least HCV CORE antigen and incubating the resultant mixture for a time and under conditions sufficient to form antigen/antibody complexes;

b. contacting the complexes with a probe comprising an enhancer compound conjugated to mammalian anti-human IgM to form a second mixture reaction product;

c. contacting the second mixture reaction product with an indicator reagent comprising a signal generating compound conjugated to an enhancer compound binding member and incubating the resultant mixture for a time and under conditions sufficient for an indicator reagent reaction product; and

d. detecting the signal generated as an indication of the presence and/or amount of HCV IgM present in the test sample.

2. The assay of claim 1 wherein said HCV antigen further comprises HCV 33c antigen, HCV c-100 antigen, or any combination thereof.

3. The assay of claim 1 wherein said HCV antigen of step (a) is attached to a solid phase.

4. The assay of claim 3 wherein said solid phase is selected from the group consisting of polymeric or glass beads, microparticles, wells of a reaction tray, test tubes, nitrocellulose strips and magnetic beads.

5. The assay of claim 1 wherein said signal generating compound is selected from the group consisting of an enzyme, a luminescent compound, a chromogen, a radioactive element and a chemiluminescent compound.

6. The assay of claim 5 wherein said enzyme is selected from the group consisting of alkaline phosphatase, horseradish peroxidase and beta-galactosidase.

7. The assay of claim 1 wherein subjecting the test sample to conditions sufficient to block the effect of rheumatoid factor comprises contacting the test sample with a quantity of anti-human IgG to form a mixture, incubating the mixture for a time and under conditions sufficient to form a reaction mixture product substantially free of rheumatoid-like substance.

8. The method of claim 1 wherein the amount of HCV IgM present in the test sample is proportional to the generated signal.

9. An assay for determining the presence and/or amount of IgM to Hepatitis C Virus (HCV) which may be present in a test sample, comprising:

a. subjecting the test sample to conditions sufficient to block the effect, if any, of rheumatoid factor-like substances which may be present in the test sample and contacting the test sample with HCV antigen comprising at least HCV CORE antigen, and incubating the resultant mixture for a time and under conditions sufficient to form antigen/antibody complexes;

b. contacting the complexes with an indicator reagent comprising a signal generating compound conjugated to anti-human IgM and incubating the resultant mixture for a time and under conditions sufficient for a reaction to occur; and

c. detecting the signal generated as an indication of the presence and/or amount of HCV IgM present in the test sample, wherein the amount of HCV IgM present in the test sample is proportional to the signal generated.

10. The assay of claim 9 wherein said HCV antigen further comprises HCV 33c antigen, HCV c-100 antigen, or any combination thereof.

11. The assay of claim 9 wherein said HCV antigen of step (a) is attached to a solid phase.

12. The assay of claim 11 wherein said solid phase is selected from the group consisting of polymeric or glass beads, microparticles, wells of a reaction tray, test tubes, nitrocellulose, and magnetic beads.

13. The assay of claim 9 wherein said signal generating compound is selected from the group consisting of an enzyme, a luminescent compound, a chromogen, a radioactive element and a chemiluminescent compound.

14. The assay of claim 13 wherein said enzyme is selected from the group consisting of alkaline phosphatase, horseradish peroxidase and beta-galactosidase.

15. The assay of claim 9 wherein subjecting the test sample to conditions sufficient to block the effect of rheumatoid factor comprises contacting the test sample with a quantity of anti-human IgG to form a mixture, incubating the mixture for a time and under conditions sufficient to form a reaction mixture product substantially free of rheumatoid-like substance.

16. An assay for determining the presence and/or amount of IgM to Hepatitis C Virus (HCV) which may be present in a test sample, comprising:

a. subjecting the test sample to conditions sufficient to block the effect, if any, of rheumatoid factor-like substances which may be present in the test sample and contacting the test sample with anti-human IgM, and incubating the resultant mixture for a time and under conditions sufficient to form resultant complexes;

b. contacting the complexes with an indicator reagent comprising a signal generating compound conjugated to HCV antigen comprising at least HCV CORE antigen and incubating the resultant mixture for a time and under conditions sufficient for a reaction to occur; and

c. detecting the signal generated as an indication of the presence and/or amount of HCV IgM present in the test sample, wherein the amount of HCV IgM present in the test sample is proportional to the signal generated.

17. The assay of claim 16 wherein said HCV antigen further comprises HCV 33c antigen, HCV c-100 antigen, or any combination thereof.

18. The assay of claim 16 wherein said anti-human IgM of step (a) is attached to a solid phase.

19. The assay of claim 18 wherein said solid phase is selected from the group consisting of polymeric or glass beads, microparticles, wells of a reaction tray, test tubes, nitrocellulose, and magnetic beads.

20. The assay of claim 16 wherein said signal generating compound is selected from the group consisting of an enzyme, a luminescent compound, a chromogen, a radioactive element and a chemiluminescent compound.

21. The assay of claim 16 wherein subjecting the test sample to conditions sufficient to block the effect of rheumatoid factor comprises contacting the test sample with a quantity of anti-human IgG to form a mixture, incubating the mixture for a time and under conditions sufficient to form a reaction mixture product substantially free of rheumatoid-like substance.

22. An assay for determining the presence and/or amount of IgM to Hepatitis C Virus (HCV) which may be present in a test sample, comprising:

a. subjecting the test sample to conditions sufficient to block the effect, if any, of rheumatoid factor-like substances which may be present in the test sample and contacting the test sample with anti-human IgM, and incubating the resultant mixture for a time and under conditions sufficient to form resultant complexes;

b. contacting the complexes with HCV antigen comprising at least HCV CORE antigen and incubating the resultant mixture for a time and under conditions sufficient for a reaction product to form;

c. contacting the reaction product with an indictor reagent comprising anti-HCV CORE antigen conjugated a signal generating compound, and incubating for a time and under conditions for a reaction to occur; and

c. detecting the signal generated as an indication of the presence and/or amount of HCV IgM present in the test sample, wherein the amount of HCV IgM present in the test sample is proportional to the signal generated.

23. The assay of claim 22 wherein said HCV antigen further comprises HCV 33c antigen, HCV c-100 antigen, or any combination thereof.

24. The assay of claim 23 wherein the indictor reagent further comprises anti-HCV 33c, anti-HCV c-100, or any combination thereof.

25. The assay of claim 22 wherein said anti-human IgM of step (a) is attached to a solid phase.

26. The assay of claim 22 wherein said solid phase is selected from the group consisting of polymeric or glass beads, microparticles, wells of a reaction tray, test tubes, nitrocellulose, and magnetic beads.

27. The assay of claim 22 wherein said signal generating compound is selected from the group consisting of an enzyme, a luminescent compound, a chromogen, a radioactive element and a chemiluminescent compound.

28. The assay of claim 22 wherein subjecting the test sample to conditions sufficient to block the effect of rheumatoid factor comprises contacting the test sample with a quantity of anti-human IgG to form a mixture, incubating the mixture for a time and under conditions sufficient to form a reaction mixture product substantially free of rheumatoid-like substance.

29. An assay for determining the presence and/or amount of IgM to Hepatitis C Virus (HCV) which may be present in a test sample, comprising:

a. subjecting the test sample to conditions sufficient to block the effect, if any, of rheumatoid factor-like substances which may be present in the test sample and contacting the test sample with mammalian anti-human IgM and incubating the resultant mixture for a time and under conditions sufficient to form antigen/antibody complexes;

b. contacting the complexes with a probe comprising an enhancer compound conjugated to HCV antigen comprising at least HCV CORE antigen to form a second mixture reaction product;

c. contacting the second mixture reaction product with an indicator reagent comprising a signal generating compound conjugated to an enhancer compound binding member and incubating the resultant mixture for a time and under conditions sufficient for an indicator reagent reaction product; and

d. detecting the signal generated as an indication of the presence and/or amount of HCV IgM present in the test sample.

30. The assay of claim 29 wherein said HCV antigen further comprises HCV 33c antigen, HCV c-100 antigen, or any combination thereof.

31. The assay of claim 29 wherein said anti-human IgM of step (a) is attached to a solid phase.

32. The assay of claim 31 wherein said solid phase is selected from the group consisting of polymeric or glass beads, microparticles, wells of a reaction tray, test tubes, nitrocellulose strips and magnetic beads.

33. The assay of claim 29 wherein said signal generating compound is selected from the group consisting of an enzyme, a luminescent compound, a chromogen, a radioactive element and a chemiluminescent compound.

34. The assay of claim 29 wherein subjecting the test sample to conditions sufficient to block the effect of rheumatoid factor comprises contacting the test sample with a quantity of anti-human IgG to form a mixture, incubating the mixture for a time and under conditions sufficient to form a reaction mixture product substantially free of rheumatoid-like substance.

35. The method of claim 29 wherein the amount of HCV IgM present in the test sample is proportional to the generated signal.

36. A test kit useful for determining the presence and/or amount of HCV IgM in a test sample, comprising:

a. a container containing at least one HCV antigen selected from the group consisting of HCV CORE, HCV 33c and HCV c-100;

b. a container containing an amount of anti-human IgG; and

c. a container containing an amount of mammalian anti-human IgM.

37. The kit of claim 36 wherein said HCV antigen is attached to a solid phase selected from the group consisting of polymeric or glass beads, microparticles, wells of a reaction tray, test tubes, nitrocellulose and magnetic beads.

38. The kit of claim 36 wherein said mammalian anti-human IgM is attached to a signal generating compound.

39 The kit of claim 38 wherein said signal generating compound is selected from the group consisting of an enzyme, a luminescent compound, a radioactive element and a chemiluminescent compound.

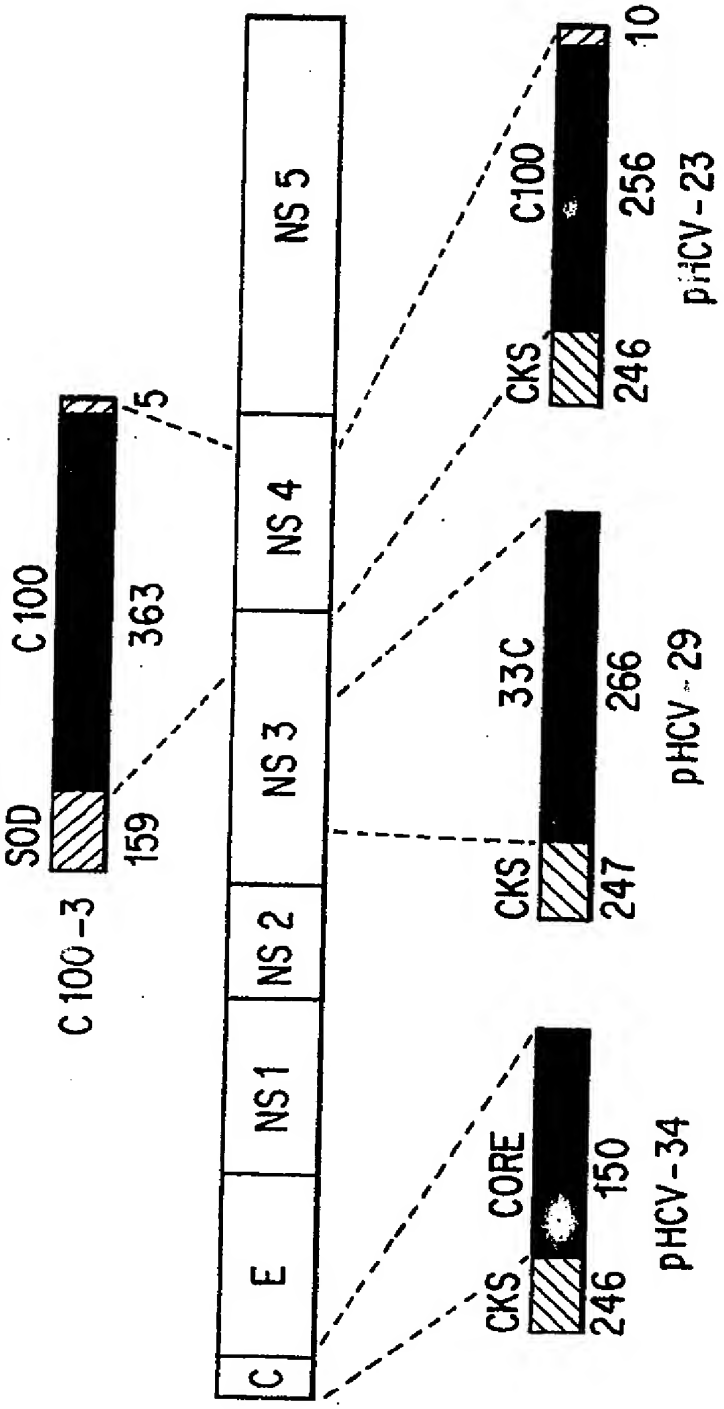
40. The kit of claim 36 wherein said mammalian anti-human IgM is attached to an enhancer compound.

41. The kit of claim 40 wherein said enhancer compound is selected from the group consisting of biotin, anti-biotin and avidin.

42. The kit of claim 41 further comprising a signal generating compound attached to an enhancer compound binding member.

43. The kit of claim 42 wherein said signal generating compound is selected from the group consisting of an enzyme, a luminescent compound and a chemiluminescent compound.

44. The kit of claim 36 wherein said enhancer compound binding member is selected from the group consisting of biotin, anti-biotin and avidin.



HCV AA#

CORE	1-150
33C	1192-1457
C100	1677-1931
C100-3	1569-1931

FIG. 1

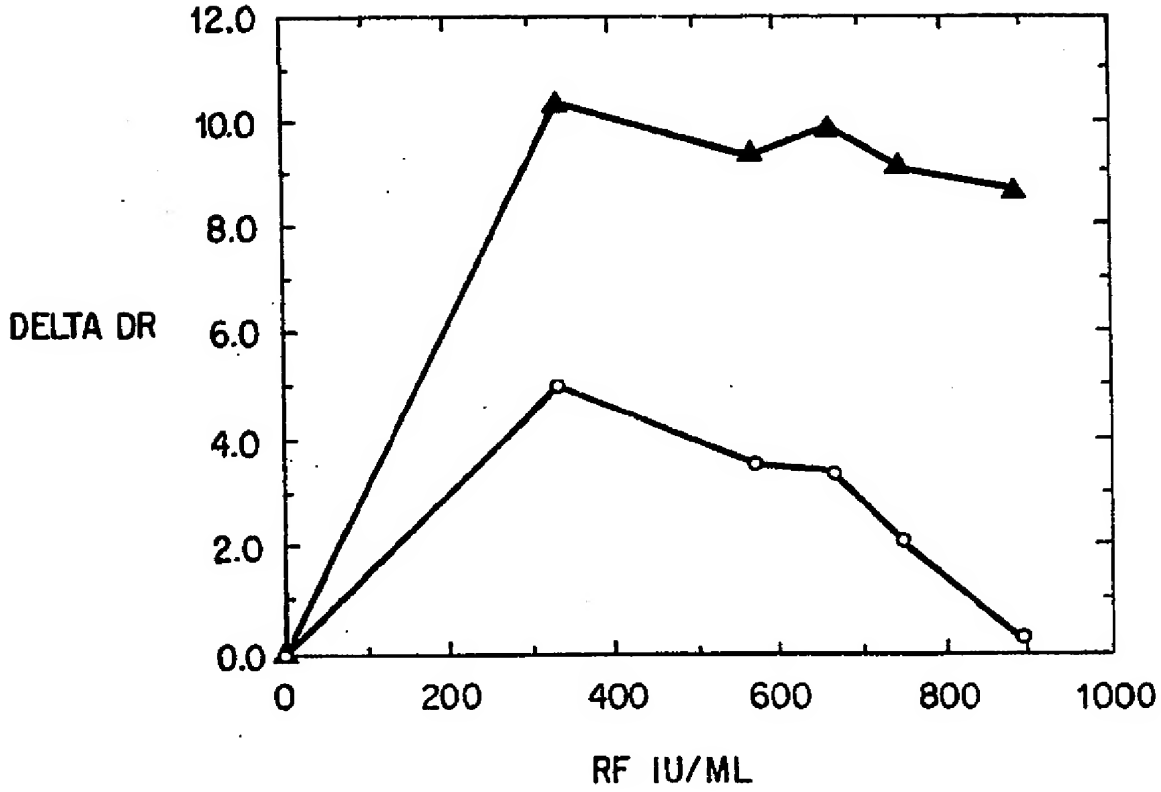


FIG. 2

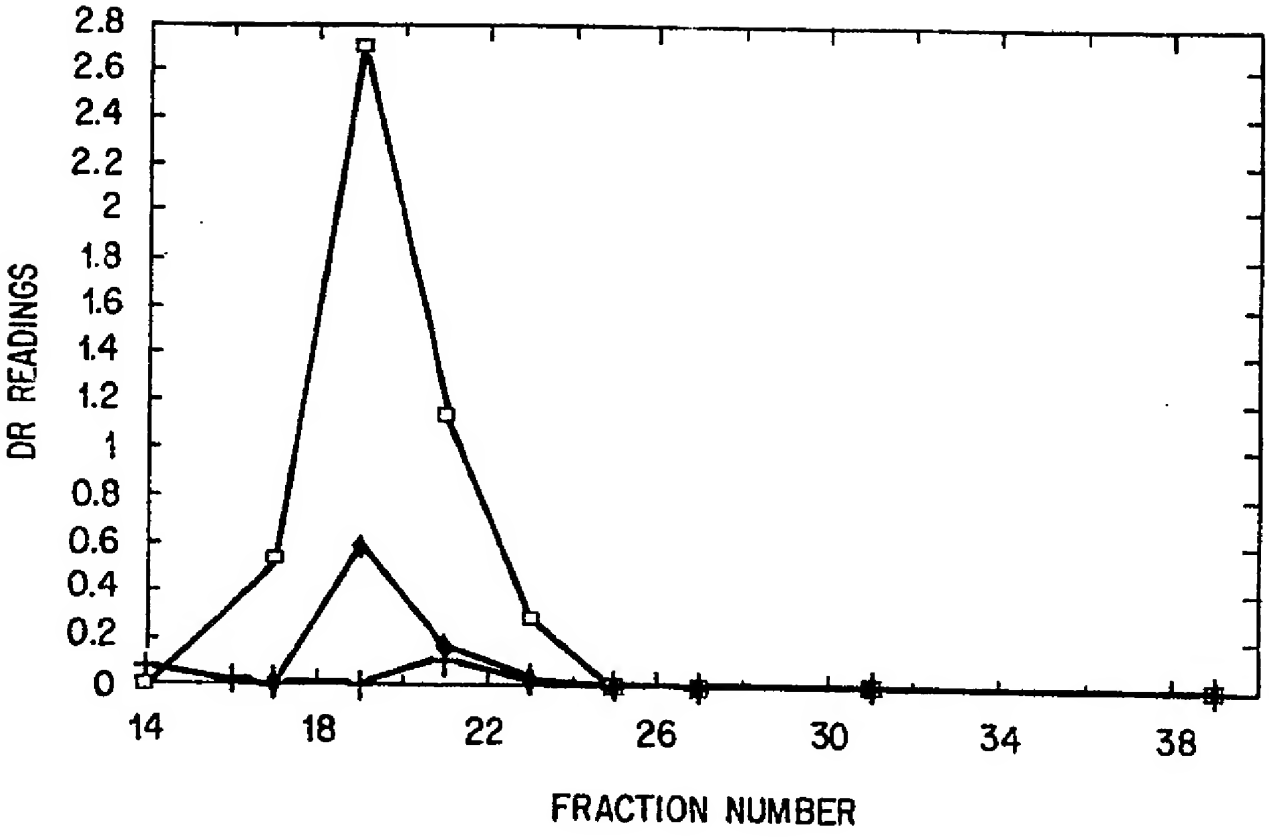


FIG. 3

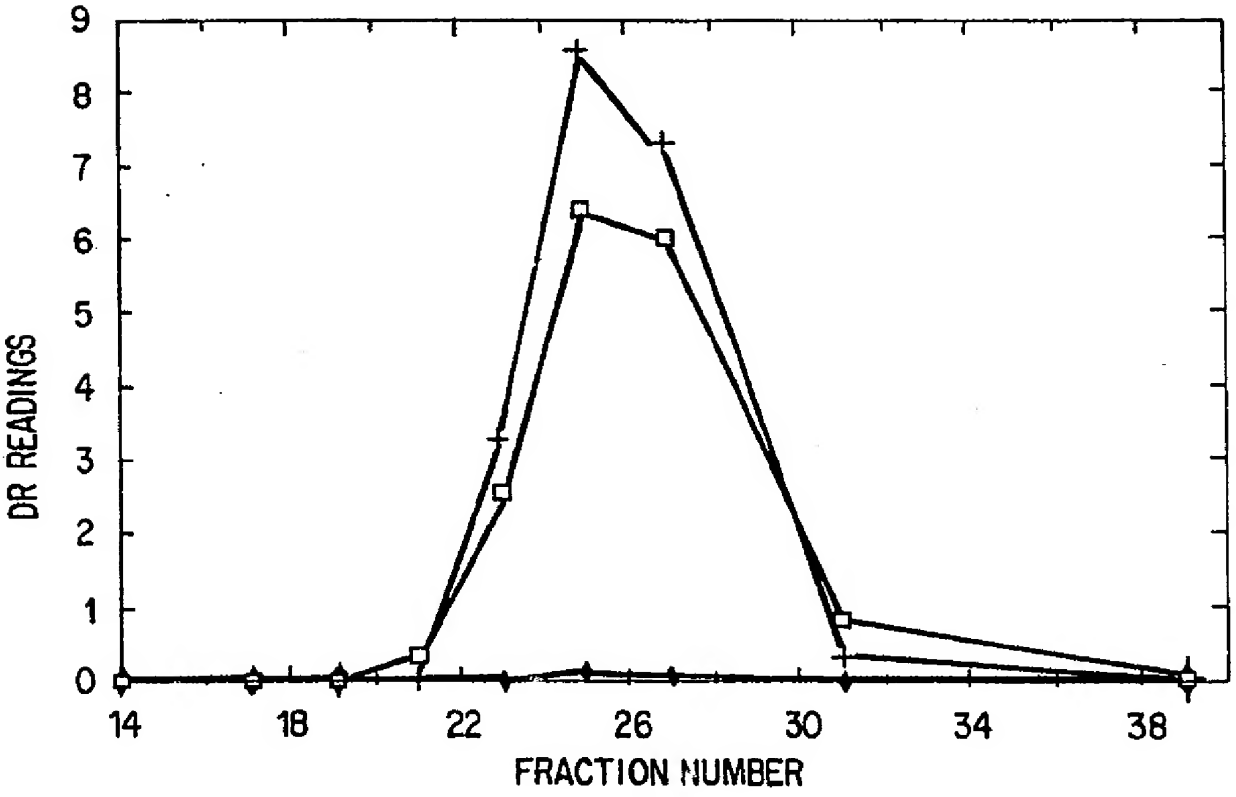


FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02523

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): G01N 33/563, 33/576, 33/537, C12Q 1/70
US CL : 436/513, 435/5

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	436/513, 435/5, 7.5, 7.9, 7.94

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

CA, Medline, Inpadoc
Search terms: Hepatitis C or HCV

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	Journal of Clinical Microbiology, Vol. 29, No. 3, issued March 1991, DAWSON ET AL, "Detection of Antibodies to Hepatitis C virus in U.S. Blood Donors," pages 551-556, see entire document.	1-44
Y	Biochemical and Biophysical Research Communications, Vol. 1 , No. 2, issued 30 October 1990, MURAISO ET AL., "A Structural Protein of Hepatitis C Virus Expressed in E. Coli. Facilitates Accurate Detection of Hepatitis C Virus," pages 511-516, see entire document.	
Y	Proceedings National Academy of Sciences (USA), Vol. 87, issued August 1990, SHIMIZU ET AL., "Early events in Hepatitis C Virus Infection of Chimpanzees," pages 6441-6444, see entire document.	1-44

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
19 June 1992	02 JUL 1992
International Searching Authority[1]	**Signature of Authorized Officer[20]**
ISA/US	M.P. WOODWARD

Form PCT/ISA/210 (second sheet)(May 1986) ß

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Journal of Clinical microbiology, Vol. 27, No. 5, issued May 1989, HO et al., "Rapid Diagnosis of Acute Epstein-Barr Virus Infection by an Indirect Enzyme Linked Immunosorbent Assay for Specific Immunoglobulin M. (lgM) Antibody without rheumatoid factor and specific lgG interference," pages 952-958, see entire document.	1-44
Y	Lancet, issued 02 June 1990, THEILMANN ET AL., "False-positive anti-HCV tests in rheumatoid arthritis," page 1346, see entire document.	1-44

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.